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Original Articles

Human telomerase reverse transcriptase depletion potentiates the growth-inhibitory activity of imatinib in chronic myeloid leukemia stem cells

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ABSTRACT

Although tyrosine kinase inhibitors (TKIs) revolutionized the management of chronic myeloid leukemia (CML), resistance against TKIs and leukemia stem cell (LSC) persistence remain a clinical concern. Therefore, new therapeutic strategies combining conventional and novel therapies are urgently needed. Since telomerase is involved in oncogenesis and tumor progression but is silent in most human normal somatic cells, it may be an interesting target for CML therapy by selectively targeting cancer cells while minimizing effects on normal cells. Here, we report that hTERT expression is associated with CML disease progression. We also provide evidence that hTERT-deficient K-562 cells do not display telomere shortening and that telomere length is maintained through the ALT pathway. Furthermore, we show that hTERT depletion exerts a growth-inhibitory effect in K-562 cells and potentiates imatinib through alteration of cell cycle progression leading to a senescence-like phenotype. Finally, we demonstrate that hTERT depletion potentiates the imatinib-induced reduction of the $ALDH^+$-LSC population. Altogether, our results suggest that the combination of telomerase and TKI should be considered as an attractive strategy to treat CML patients to eradicate cancer cells and prevent relapse by targeting LSCs.

1. Introduction

Targeting break point cluster region-Abelson (BCR-ABL) activity by tyrosine kinase inhibitors (TKIs) is efficiently inducing disease remission and prolonging survival in chronic myeloid leukemia (CML), especially in newly diagnosed patients. Nevertheless, about 15% of patients fail to reach a complete cytogenetic response (CCR) and 15–20% of patients achieve CCR before losing it due to intrinsic or acquired TKI resistance and the persistence of TKI-insensitive quiescent leukemia stem cells (LSCs) [1,2]. Thus, a strategy combining conventional and novel therapies is required to overcome relapse in CML patients. In this context, telomerase inhibition could represent an effective therapeutic strategy against CML [3].

Two mechanisms maintain telomere homeostasis in cells exhibiting infinite replicative potential: telomerase activation or the alternative lengthening of telomeres (ALT) mechanism [4]. Telomerase is a ribonucleoprotein enzyme complex responsible for the synthesis of telomeric DNA repeats (TTAGGG) at the end of chromosomes, thus maintaining telomere length after DNA replication and overcoming the end replication problem. This complex consists of the human telomerase reverse transcriptase (hTERT) catalytic subunit and the human telomerase RNA gene (hTERC), which encodes an RNA component, acting as the template for the catalytic subunit for the elongation of telomeres [5]. Telomerase activity and hTERT expression are closely correlated in human cells [5,6]. Conversely to most of normal human somatic cell types with limited proliferative capacity, telomerase is upregulated in most types of cancers [7] including CML [8]. Therefore, by maintaining a stable telomere length, canonical telomerase activity is essential for long-term tumor cell proliferation.

By elongating telomeres, telomerase protects chromosome ends from being recognized as DNA damage and chromosomal rearrangements. Dysfunctional telomeres, arising by telomere critical shortening, elicit DNA damage responses (DDRs) that induce a permanent cell-cycle arrest named cellular senescence [9]. Cells entering a state of senescence are characterized by both morphological and functional alterations including an increase in senescence-associated β-galactosidase

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List of abbreviations

ALDH	aldehyde dehydrogenase
ALT	alternative lengthening of telomeres
AP	accelerated phase
APB	ALT-associated promyelocytic leukemia body
BC	blast crisis
BCR-ABL	break point cluster region-Abelson
BP	blast phase
Cas9	CRISPR associated protein 9
CCR	complete cytogenetic response
CML	chronic myeloid leukemia
CP	chronic phase
CRISPR	clustered regularly interspaced short palindromic repeats
DDR	DNA damage response
FCS	fetal calf serum
GI_{50}	growth inhibitory dose 50%
GRB2	growth factor receptor bound protein 2
GSK3	glycogen synthase kinase 3
hTERC	catalytic subunit and the human telomerase RNA gene
hTERT	human telomerase reverse transcriptase
LEF	lymphoid enhancer factor
LSC	leukemia stem cell
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase/extracellular signal-regulated kinase
PI3K	phosphoinositide 3-kinase
PML	promyelocytic leukemia
PNA	peptide nucleic acid
ROS	reactive oxygen species
RPA	anti-replication protein A
SA-β-Gal	senescence associated − β-galactosidase
sgRNA	single-guide RNA
TCF	T cell factor
TEN	TERT essential N-terminal
TFR	treatment-free remission
TKI	tyrosine kinase inhibitor
TRF2	telomeric repeat binding factor 2
WT	wild-type
Y177	tyrosine 177

(SA-β-Gal) activity and a flattened and enlarged cell morphology [10]. Cellular senescence acts as a barrier to tumorigenesis, thus underlying the therapeutic potential of therapy-induced senescence for cancer treatment [9].

Here, we showed that hTERT expression is associated with disease progression. We also provided evidence that hTERT depletion through genetic manipulations using clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas)9 is not sufficient to generate telomere shortening in K-562 CML cell line and that telomere length is maintained through the ALT pathway. We revealed that chemical and genetic telomerase inhibition in K-562 cells potentiates the growth-inhibitory effect of imatinib. This anti-proliferative effect was associated with changes in cell cycle related-proteins expression, delayed cell cycle progression and a senescence-like phenotype induction. Finally, we pointed out that hTERT depletion reduces aldehyde dehydrogenase (ALDH) expression in K-562 cells and sensitizes $ALDH^+$ cells to imatinib treatment. Altogether, our results reveal that targeting telomerase could represent an effective strategy to improve the efficacy of TKIs currently used to treat CML patients.

2. Material and methods

2.1. Association between hTERT expression and CML stages

Expression and disease phase data in patients were retrieved from the GSE4170 dataset (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE4170). Accelerated phase (AP) with cytogenetic alterations (AP cyto) were grouped with AP samples, as done originally [11]. Chronic phase (CP) samples treated with imatinib were considered as a separate group.

2.2. Cell culture and treatments

K-562 and MEG-01 cells were purchased from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSZM; Braunschweig, Germany). KBM-5 cells were a kind gift of B.B. Aggarwal (Houston, Texas). K-562 and MEG-01 cell lines were cultured in RPMI 1640 (Lonza, Verviers, Belgium), supplemented with 10% fetal calf serum (FCS; Lonza) and 1% antibiotic–antimycotic (Lonza). KBM-5 cells were cultured in IMDM (Lonza) supplemented with 10% FCS and 1% antibiotic–antimycotic. Compounds used in the study are listed in Table S1.

2.3. Generation of hTERT-deficient K-562 cell lines through CRISPR-Cas9 genome editing

Using lipofectamine (Thermofisher, Erembodegen, Belgium), K-562 cells were transfected with an all-in-one vector simultaneously expressing Cas9-GFP and single-guide RNA (sgRNA) targeting the first exon of hTERT gene (Sigma-Aldrich, Bornem, Belgium) (sequence: 5′-CGGTAGTGGCTGCGCAGCAGGG-3′). This exon codes for the TERT essential N-terminal (TEN) domain, which is essential for telomere interaction and synthesis [12] (Fig. 1). Generation of hTERT-deficient K-562 cell lines and methods used for sequencing are detailed in the Supplementary Materials and Methods.

2.4. Telomerase activity assay

Telomerase activity was measured as previously described [13].

2.5. Relative telomere length

Relative telomere length was determined by flow-fluorescent *in situ* hybridization with a fluorescein-conjugated peptide nucleic acid (PNA) probe using the Telomere PNA Kit/FITC (Dako Denmark A/S, Heverlee,

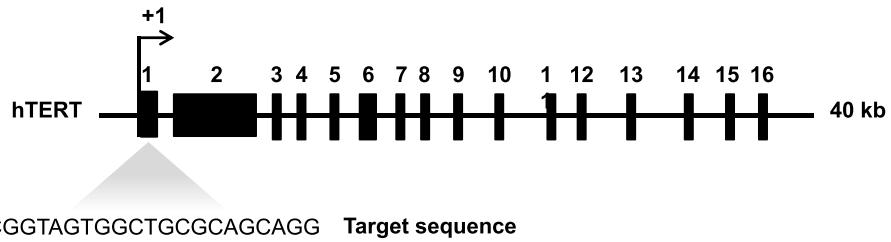


Fig. 1. Schematic representation of hTERT gene and the sgRNA target. Exon-intron organization of hTERT gene is shown. Coding exons are represented by boxes and lines connecting the boxes are introns. The sgRNA targeting site is located within the first exon coding for the TEN domain essential for telomere interaction and synthesis.

Belgium) according to the manufacturer's protocol. Relative telomere length was quantified by flow cytometry.

2.6. Immunofluorescence microscopy

To detect ALT-associated promyelocytic leukemia (PML) bodies (APBs), cells were stained with anti-telomeric repeat binding factor 2 (TRF2) antibody, and anti RAD51, anti-replication protein A (RPA), or anti-PML antibodies. Detailed methods are described in the Supplementary Materials and Methods.

Lysosomal cellular distribution was assessed as previously reported [13].

2.7. Assessment of cell growth inhibition

Cell proliferation was assessed by light microscopy and trypan blue exclusion test after staining with 0.4% trypan blue solution (Sigma-Aldrich). Growth inhibitory dose 50% (GI_{50}) was calculated by a non-linear regression using the GraphPad Prism 8.0 software (La Jolla, CA, USA).

2.8. Colony formation assays

Colony formation assays were carried out as previously described elsewhere [14] with a seeding concentration of 10^3 cells/mL. Colonies were detected after 12 days of culture.

2.9. Cell cycle analysis

Cell cycle distribution was analyzed as previously described [15].

2.10. Protein extraction and Western Blot

Protein extraction and Western Blots were carried out as previously described [16]. Antibodies used are listed in Table S2. Band intensities were quantified using ImageQuant TL (GE Healthcare, Buckinghamshire, UK) and values of fold change relative to loading control are indicated underneath Western Blots.

2.11. SA-β-Gal assay

SA-β-Gal activity was measured as previously reported [17]. K-

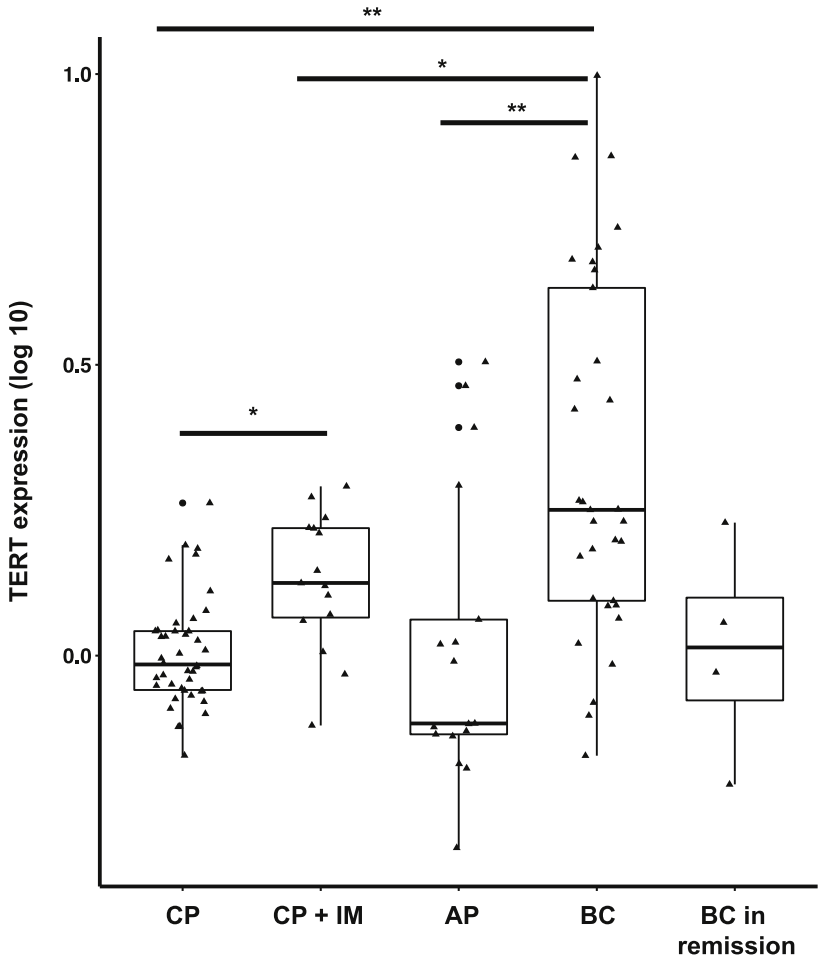


Fig. 2. hTERT expression is higher in blast crisis phase. Boxplots of hTERT expression values in CML patients normalized to a chronic phase (CP) sample pool in the different phase groups: accelerated phase (AP), blast crisis (BC) and CP. Circles and triangles represent outliers and patients, respectively. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively (p-value from pairwise Welch *t*-tests, adjusted using Holm P-value adjustment). IM: imatinib.

562 cells treated with 80 nM doxorubicin for 72 h were used as a positive control for senescence induction.

2.12. Detection of ALDH$^+$ cells

ALDEFLUOR™ kit (STEMCELL Technologies) was used to determine the percentage of ALDH$^+$ cells. Cells were labeled following manufacturer's instructions and then processed using flow cytometry.

2.13. Flow cytometry acquisition and analysis

Flow cytometry acquisitions were performed on a FACSCalibur (Becton Dickinson Biosciences) using CellQuest software (Becton Dickinson Biosciences). Data were analyzed using FlowJo 10 software (Treestar, Ashland, USA).

2.14. Statistical analysis

Statistical analyses were performed using the GraphPad Prism 8.0 software. For analyzing the association between hTERT expression and CML stages, considering that the design was heavily unbalanced, statistical analysis was performed using pairwise *t* tests with p-value adjustment, not considering a pooled standard deviation (Welch tests). One-way ANOVA followed by the Dunnett's multiple comparison tests, Student's *t*-test or Welch *t*-test were used for statistical comparisons. P-values below 0.05 were considered as statistically significant.

3. Results

3.1. Increased hTERT expression is associated with CML progression

We first investigated whether hTERT expression level is a prognostic factor of CML disease progression. As shown in Fig. 2, hTERT

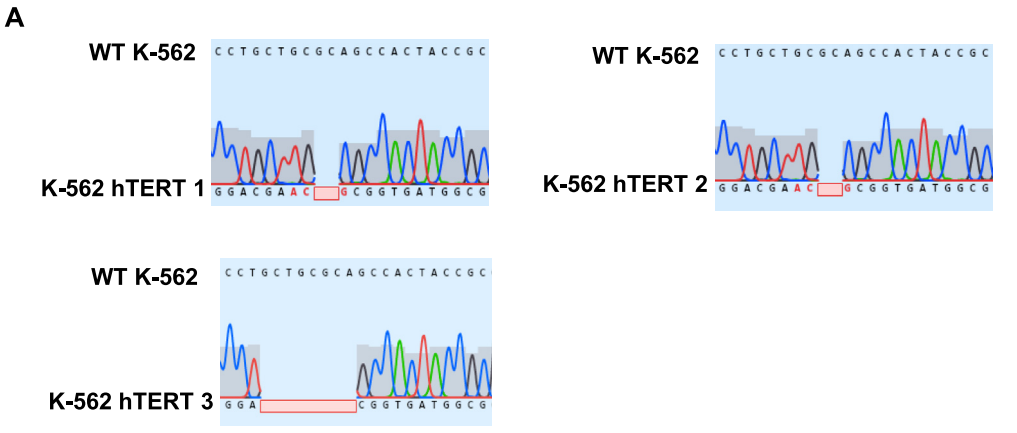
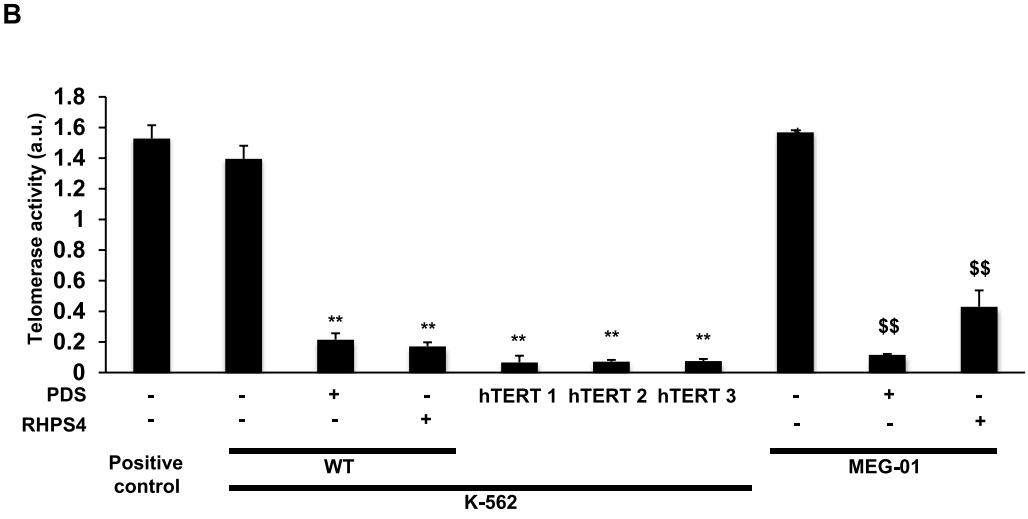


Fig. 3. Generation of hTERT-deficient K-562 cell lines by CRISPR-Cas9-mediated genome editing. (A) Upper panel: alignment of Sanger sequencing confirmed insertion-deletion mutations (red boxes) in the sgRNA target sequence in hTERT-deficient K-562 cells compared to WT K-562 cells. Lower panel: alignment of hTERT protein sequences corresponding to exon 1. Frameshift mutations are indicated in red and the sgRNA target sequence is underlined. (B) Telomerase activity measurement. Pyridostatin (PDS) and RHPS4 were used as reference chemical inhibitors of telomerase activity. K-562 and MEG-01 cells were treated for 72 h with 25 μM and 20 μM PDS, or 12 μM and 40 μM RHPS4, respectively. Data are the mean ± SD of at least four independent experiments. * and ** indicate $p < 0.05$ and $p < 0.01$ *versus* WT K-562 cells, respectively (one-way ANOVA, $p < 0.001$); $$ indicates $p < 0.01$ *versus* MEG-01 cells (Student's *t*-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



expression is significantly upregulated in patients in blast crisis (BC) compared to patients in CP or AP ($p = 9.8e^{-06}$ and $p = 0.0032$, respectively). In addition, imatinib-treated patients in CP significantly display higher hTERT expression levels compared to untreated patients. These results suggest that hTERT expression is associated with disease progression in CML patients, supporting the hypothesis that telomerase represents a therapeutic target.

3.2. Generation of hTERT-deficient K-562 cell lines by CRISPR-Cas9-mediated genome editing

hTERT-deficient K-562 cell lines were generated using CRISPR-Cas9-mediated genome editing. Three cell lines (K-562 hTERT 1, 2 and 3) presenting a deletion of hTERT compared to wild type (WT) K-562 cells (Fig. 3A, upper panel) were selected. K-562 hTERT 1 and 2 cells harbored a 3-bp deletion as well as a 1-bp insertion in the target sequence in exon 1, whereas K-562 hTERT 3 cells exhibited an 8-bp deletion in the target sequence. These deletions allowed predicting frameshift mutations resulting in a mutant hTERT protein (Fig. 3A, lower panel).

As shown in Fig. 3B, K-562 hTERT 1, 2 and 3 cells exhibit no telomerase activity, similarly to K-562 and MEG-01 cells treated with the G-quadruplex chemical ligands pyridostatin and RHPS4, which display a significant inhibition of telomerase activity in contrast to untreated cells.

3.3. hTERT-deficient K-562 cells maintain their telomere length through the ALT pathway

We evaluated the impact of hTERT depletion on telomere length in K-562 cells (Fig. 4A). Flow cytometry analyses of relative telomere length indicated that, although telomerase activity was abrogated in hTERT-deficient K-562 cells, telomeres were not shortened, as expected. Therefore, we evaluated whether hTERT-deficient K-562 cells rely on the ALT pathway. Human ALT-positive cells contain unique nuclear structures called APBs containing telomeric DNA, telomere-associated proteins such as TRF2 [18], and proteins involved in DNA recombination and repair like RAD51 and RPA, or PML [19]. Thus, we investigated whether TRF2 is co-expressed with RAD51, RPA or PML in WT and hTERT-deficient K-562 cells (Fig. 4A, B and C). Immunofluorescence staining showed that a similar percentage of cells co-expressing TRF2 with RAD51, RPA or PML proteins in WT, K-562 hTERT 1, 2 and 3 cells (Fig. 4A, B and C, lower panels). These results suggest that hTERT-deficient K-562 cells maintain their telomere length through the ALT pathway.

3.4. hTERT depletion sensitizes K-562 cells to the anti-proliferative effect of imatinib

The impact of hTERT depletion in combination with imatinib was assessed on cell proliferation in K-562 cells. As shown in Table 1, K-562 hTERT 1, 2 and 3 cells appeared more sensitive to imatinib treatment (GI_{50} = 0.17, 0.13 and 0.16 μM, respectively) compared to WT K-562 cells (GI_{50} = 0.37 μM), thus suggesting that hTERT depletion potently enhances imatinib-induced growth arrest.

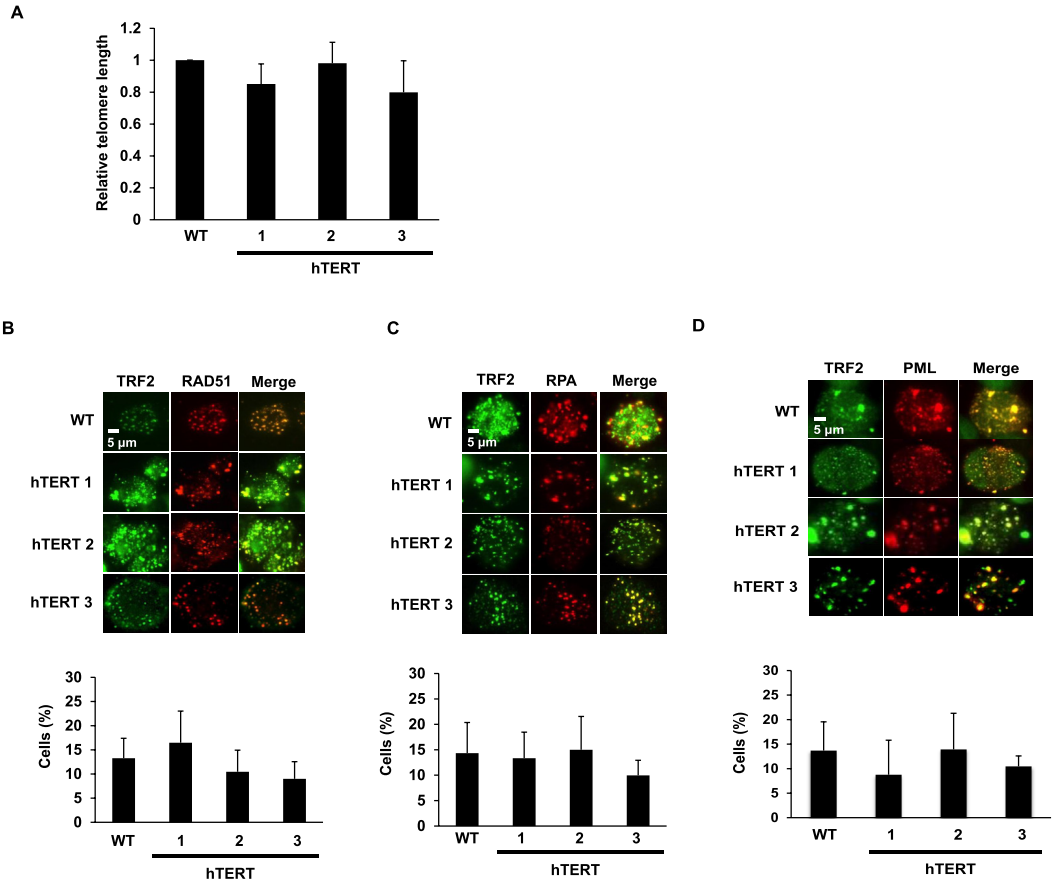


Fig. 4. hTERT-deficient K-562 cells display the hallmark of the ALT pathway. WT and hTERT-deficient K-562 cells were assayed (A) by flow cytometry for relative telomere length or (B, C, D) immunostaining for TRF2 in green and RAD51 (A), RPA (B) or PML bodies (C) in red. All pictures are representative of three independent experiments. Percentage of cells showing co-expression of TRF2 with RAD51, RPA or PML is indicated above. All graphs represent the mean ± SD of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
In vitro growth inhibitory activity of imatinib against WT and hTERT-deficient K-562 cells.

K-562 cell lines	GI_{50} (μM)[a]
WT	0.36 (± 0.04)
hTERT 1	0.18 (± 0.05) *
hTERT 2	0.09 (± 0.02) **
hTERT 3	0.12 (± 0.02) **

[a] GI_{50}: concentration of imatinib inhibiting 50% of the growth of the indicated cell line after 72 h of treatment. Results represent the mean ± SD of three independent experiments. * and ** indicate $p < 0.05$ and $p < 0.01$ *versus* WT K-562 cells, respectively (Student's *t*-test).

We further evaluated cell proliferation of WT and hTERT-deficient K-562 cells in a three-dimensional colony formation assay. As shown in Fig. 5A, the number of colonies in K-562 hTERT 1, 2 and 3 cells significantly increased by 1.5-, 2.4- and 3.1-fold respectively, compared to WT cells, thus suggesting that hTERT depletion in K-562 cells partially inhibits colony formation. We further assessed whether telomerase inhibition may potentiate the effects of imatinib on colony formation. Chemical telomerase inhibition using pyridostatin significantly decreased the number of colonies by 67% compared to WT K-562 cells and combination with imatinib totally abrogated colony formation (Fig. 5B). Similarly, hTERT depletion through genomic editing potentiated the inhibitory impact of imatinib on colony formation by 69, 72 and 76% in hTERT K-562 1, 2 and 3 cell lines, respectively (Fig. 5C, D and E). These results suggest that telomerase inhibition sensitizes WT K-562 cells to the anti-proliferative effect of imatinib.

3.5. hTERT depletion combined to imatinib alters cell-cycle kinetics in K-562 cells

We further explored whether hTERT depletion and imatinib exert their anti-proliferative effect by affecting the cell cycle. As shown in Fig. 6A, hTERT depletion changes WT K-562 cell cycle kinetics by significantly delaying the passage of cells through G2 to M phase. Indeed, 11% of WT cells *vs* 23, 27 and 24% of K-562 hTERT 1, 2 and 3 cells respectively, were arrested in G2/M phase. We then evaluated the impact of imatinib on WT and hTERT-deficient K-562 cell cycle distribution. Results revealed that imatinib significantly increased the percentage of cells in G2/M phase from 8 to 46.4% in WT K-562 cells and by 1.8- and 1.6-fold in K-562 hTERT 1 and 2 cells, respectively (Fig. 6A). Finally, imatinib triggered a 1.7-fold increase of the percentage of cells in G1 phase in K-562 hTERT 2 cells, compared to untreated cells.

To gain insight into the mechanisms leading to altered cell cycle progression, we further monitored the expression of cell cycle-related proteins. As shown in Fig. 6B, p21 levels were moderately increased in K-562 hTERT 1 and 2 cells compared to WT cells, although the constitutive protein expression level of the G1-S/G2-M phase-checkpoint regulator p27 did not change between WT and hTERT-deficient K-562 cells. We also observed that cyclin D1 levels were upregulated in K-562 hTERT 2 and 3 cells and cyclin B1 levels were increased in K-562 hTERT 1, 2 and 3 cells compared to WT cells (Fig. 6B). These results suggest that modulation of cell cycle-related protein expression is in accordance with growth inhibition associated with hTERT depletion.

We next provided evidence that imatinib upregulates the expression of p21 and p27 in WT K-562 cells. Meanwhile, imatinib triggers a moderate decrease of the expression of cyclin D1 and a drastic downregulation of c-Myc (Fig. 6B). To confirm the impact of imatinib on the expression of cell cycle-related genes in CML cells, we used the publicly available microarray dataset GSE1922. Figs. S1 and S2 and Table S3 show genes that are modulated in imatinib-treated K-562 cells. In accordance with our Western blot data, analysis of the microarray dataset confirmed that imatinib induces the upregulation of cyclin-dependent kinase inhibitors (*e.g.* p27) and downregulation of cell cycle regulators such as cyclins.

Western Blot analyses showed that c-Myc and cyclin D1 were dramatically decreased by imatinib in hTERT-deficient K-562 cells. Interestingly, hTERT 1 and 2 cells display more a pronounced increase in the level of p21 compared to WT cells following imatinib treatment. Similarly, imatinib led to a significant increase of p27 expression in K-562 hTERT 2 cells compared to imatinib-treated WT cells (Fig. 6B). This suggests that hTERT depletion potentiates the cell cycle-inhibitory effects of imatinib through p21 and p27 upregulation.

Western Blot analyses also revealed that imatinib led to a significant reduction of phosphorylated BCR-ABL levels in K-562 cells. Remarkably, phosphorylated BCR-ABL levels were almost totally abrogated in K-562 hTERT cells concomitantly with decreased BCR-ABL expression (Fig. 6B), indicating that the reduction of phosphorylated BCR-ABL levels is enhanced when imatinib was combined with hTERT depletion.

3.6. hTERT depletion combined to imatinib induces a senescence-like phenotype

Altered expression of the key cell cycle regulators p21 and p27 in imatinib-treated hTERT-deficient K-562 cells prompted us to evaluate whether cellular senescence could be triggered by telomerase inhibition combined to imatinib. K-562 cells treated with doxorubicin, a known inducer of cellular senescence [20], were used as a positive control. Doxorubicin induced a 3.9-fold increase in SA-β-Gal activity in K-562 cells (Fig. 7A and Fig. SI3A). Chemical telomerase inhibition through pyridostatin and RHPS4 triggered a 3.3 and 1.9-fold increase in SA-β-Gal activity compared to control cells, respectively (Fig. 7A, lower panel). Similarly, compared to WT cells, K-562 hTERT 1, 2 and 3 presented a 3.2, 5.3 and 5.8-fold increase in SA-β-Gal activity, respectively (Fig. 7B, lower panel and SI3B). In addition, imatinib-exposed K-562 hTERT 1, 2, 3 cells display a 1.5-, 1.7- and 1.7-fold increase in SA-β-Gal activity, respectively (Fig. 7B, lower panel). These results suggest that hTERT depletion potentiates the increase in SA-β-Gal activity induced by imatinib in K-562 cells.

Since increased intracellular reactive oxygen species (ROS) production has been demonstrated to play a key role in the induction of the cellular senescence program [21], we investigated whether increased SA-β-Gal activity associated with hTERT depletion involved enhanced intracellular ROS levels. Cytometry analyses revealed that ROS levels were not increased following hTERT depletion (Fig. SI4).

Considering that lysosome mass has been reported to be consistently enhanced in senescent cells [10,13] we further assessed lysosome distribution (Fig. 7C and D). Results pointed out a 1.6, 1.7 and 1.9-fold increase of the lysosomal mass in hTERT 1, 2 and 3 cells, respectively, compared to WT cells (Fig. 7D). In addition, hTERT depletion potentiated imatinib-mediated lysosomal mass enhancement in hTERT K-562 1, 2 and 3 cells with a 1.4-, 1.5- and 1.9-fold increase respectively, compared to imatinib-treated WT cells (Fig. 7C and D). These results indicate that the lysosomal compartment is more prominent in the different hTERT-deficient cells compared to WT K-562 cells.

Considering that persistent DDR activation could be involved in senescence onset and maintenance [10], we further analyzed the levels of γH2AX. Western Blot analyses revealed that γH2AX levels are markedly upregulated in imatinib-treated WT and hTERT-deficient K-562 cells (Fig. 7E), suggesting that the senescence-like phenotype observed in imatinib-treated cells is associated with the activation of DDR signaling.

3.7. hTERT depletion reduces the cancer stem cell population in WT and hTERT-deficient K-562 cells and sensitizes them to imatinib treatment

Since a growing body of evidence is suggesting that LSCs,

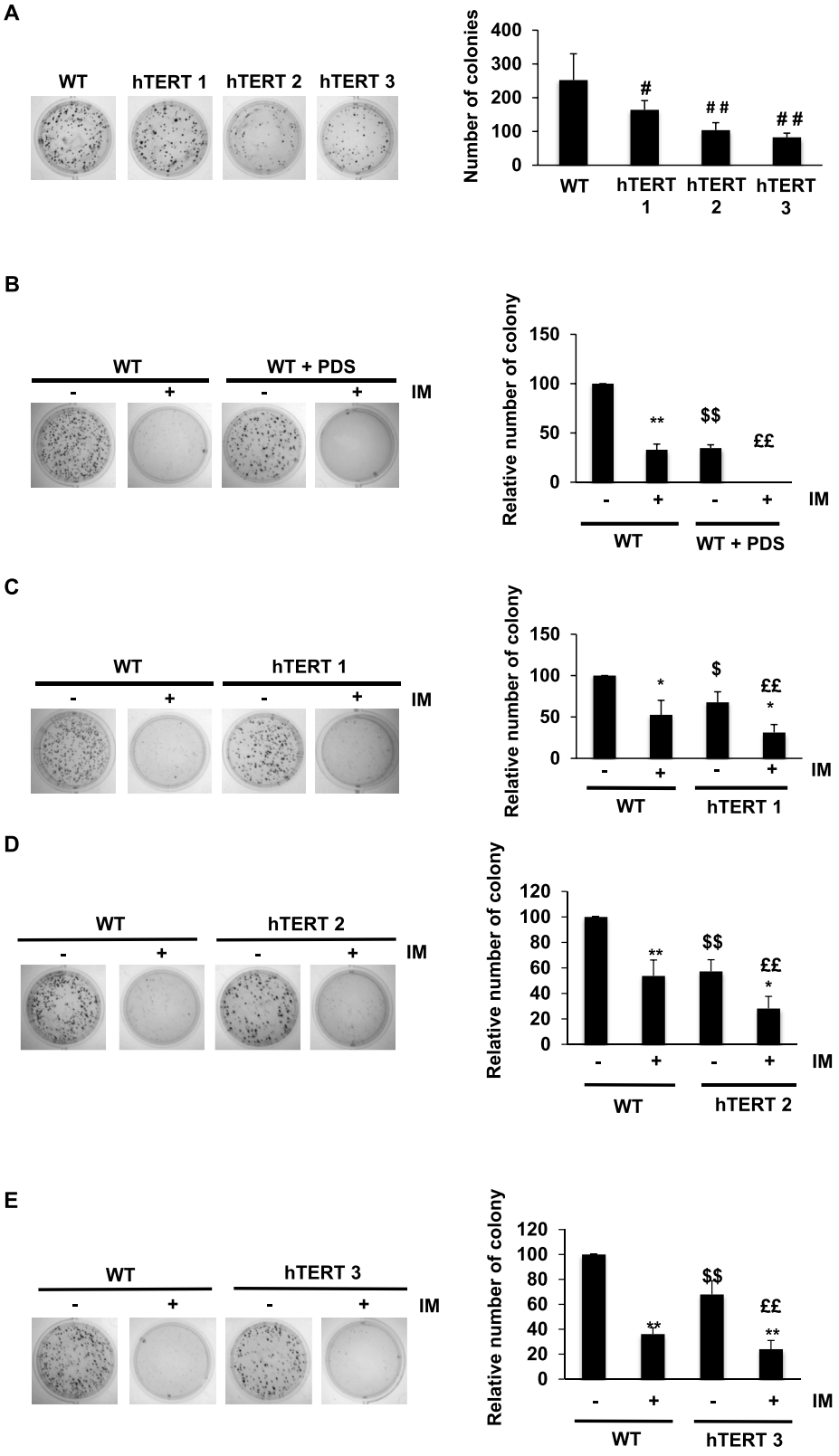


Fig. 5. hTERT depletion potentiates the growth inhibitory effects of imatinib. Colony formation assays were performed in WT and K-562 deficient cells for 12 days (A, B, C, D, E). (B) WT K-562 cells were pre-treated with 25 μM pyridostatin (PDS) for 72 h before imatinib treatment. All pictures are representative of at least three independent experiments (left panel). All graphs represent the mean of the number of colonies ± SD of at least three independent experiments (right panel). * and ** indicate $p < 0.05$ and $p < 0.01$ *versus* WT K-562 cells, respectively; £ and ££ indicate $p < 0.05$ and $p < 0.01$ *versus* untreated cells, respectively; $ and $$ indicate $p < 0.05$ and $p < 0.01$ *versus* imatinib-treated WT K-562 cells, respectively. Student's *t*-test was used for all these statistical comparisons except for comparison between WT K-562 cells *versus* PDS-treated WT K-562 cells, imatinib-treated WT K-562 cells and WT K-562 cells co-treated with PDS and imatinib, where the one-way ANOVA test was used ($p < 0.001$). # and ## indicate $p < 0.05$ and $p < 0.01$ *versus* WT K-562 cells, respectively.

characterized by elevated ALDH activity levels, play a critical role in TKI resistance and CML relapse [22], we further tested whether hTERT depletion could impact ALDH expression in K-562 cells. Results demonstrated that ALDH$^+$ cells represent 48% of total cell population in K-562 cell line, whereas this fraction is reduced to 23.1, 29 and 21.1% in K-562 hTERT 1, 2 and 3 cells, respectively (Fig. 8A and B).

Then, we investigated the impact of imatinib on ALDH expression in the different cell lines. We found that imatinib triggers a significant 1.4-fold decrease in WT K-562 cells. Additionally, the percentage of ALDH$^+$ cells was reduced in imatinib-treated K-562 hTERT 1, 2 and 3 cells by 4.4, 16.5 and 2-fold, respectively, compared to imatinib-treated WT cells (Fig. 8A and B). These results suggest that hTERT depletion sensitizes ALDH$^+$ cells to imatinib treatment in K-562 cells.

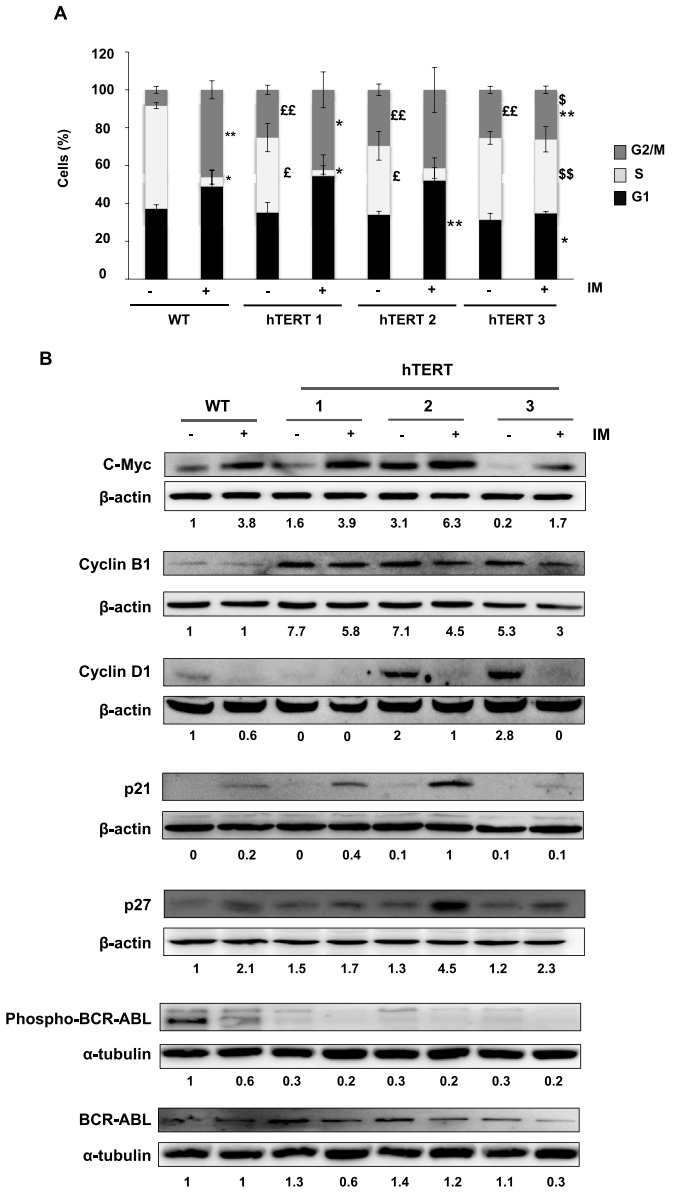


Fig. 6. hTERT depletion combined to imatinib alters cell cycle distribution. Cell lines treated with their respective GI_{50} values of imatinib (IM; see Table 1) for 72 h were assessed for (A) cell cycle distribution and (B) the expression of cell cycle related proteins by Western blot. Values of fold change relative to loading control are indicated under blots. (After quantification, untreated WT K562 were set as 1, besides for p21 expression where imatinib-treated hTERT K-562 2 cells were set as 1 as p21 expression levels were undetectable in WT K562). Blots are representative results of three independent experiments. Results represent the mean ± SD of 3 independent experiments. * and ** indicate $p < 0.05$ and $p < 0.01$ *versus* untreated cells, respectively (Student's *t*-test); £ and ££ indicate $p < 0.05$ and $p < 0.01$ *versus* WT K-562 cells, respectively (one-way ANOVA; $p = 0.2$, $p < 0.008$ and $p < 0.0001$ for G1, S and G2/M phase, respectively); $ and $$ indicate $p < 0.05$ and $p < 0.01$ *versus* imatinib-treated WT K-562 cells, respectively (one-way ANOVA; $p = 0.08$, $p < 0.001$ and $p = 0.009$ for G1, S and G2/M phase, respectively).

4. Discussion

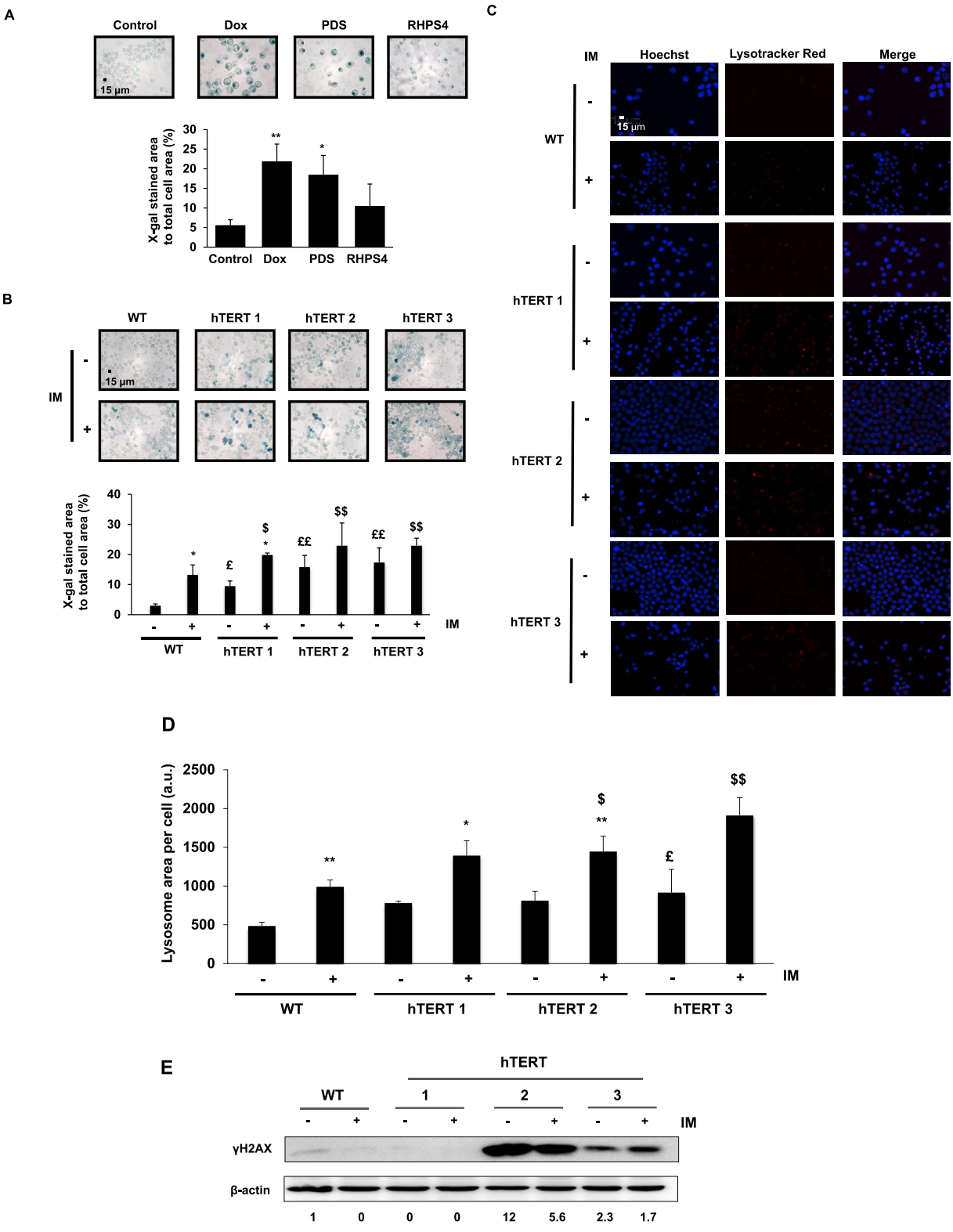
Several anti-telomerase therapeutics including the oligonucleotide inhibitor imetelstat (GRN163L) and telomerase vaccines have shown effective anti-cancer responses in a wide range of cancers [23,24], but impact of telomerase inhibitors in CML has not yet been reported.

Since hTERT is considered as the limiting factor for telomerase enzymatic activity [25], we studied the expression of this gene among the different stages of CML. We reported that hTERT expression is up-regulated in patients with BC compared to patients in CP or AP highlighting that expression of this gene is associated with disease progression. These results are in accordance with other reports that show increased levels of telomerase activity in CML patients during disease progression [26,27]. In addition, hTERT expression was reported to differ among the different stages of CML and is higher in patients in BP compared to CP and AP although results appeared not significant [28].

In human cancer cells and immortalized cell lines, telomere shortening may be compensated either by telomerase up-regulation or by ALT that is defined as a telomerase-independent mechanism involving DNA repair and recombination processes [4]. 15% of all cancers maintain telomere length homeostasis through this process based on homologous recombination [29]. ALT-mediated telomere regulation has been suggested to also occur in CML [30]. ALT^+ cells typically exhibit APBs that are specialized PML bodies colocalizing with DNA repair and recombination proteins. APBs are found in a small subset of cells (approximately 5–10%) of cells within asynchronously dividing ALT cell populations [18]. Here, we found that K-562 cells display both telomerase activity and APB structures in a small subset of cells. The coexistence of these two pathways of telomere maintenance in different cancer populations or within the same cell was reported, in agreement with our data. Indeed, telomerase-positive breast cancer cells were demonstrated to present hallmarks of the ALT pathway [31]. In addition, ALT-positive cells reconstituted for telomerase activity maintain their telomeres with both pathways of telomere maintenance [32–34]. We also provided evidence that hTERT-deficient K-562 cells do not display telomere shortening and present APB structures, thereby suggesting that telomere length might be maintained through the ALT pathway. These results are in line with other studies reporting that telomerase inhibition triggers a switch from telomerase activity to the ALT mechanism to maintain telomere elongation [35,36].

Here, we reported that hTERT depletion exerts growth-inhibitory effects in K-562 cells although telomeres are not shortened. There is a large body of evidence suggesting that hTERT promotes cell growth and proliferation through telomere length–independent mechanisms [37–39]. Specifically, hTERT expression in mouse skin promotes epithelial proliferation through transcriptional control of a c-Myc- and Wnt-related developmental program [40]. In addition, hTERT was found to modulate c-Myc ubiquitination and proteasomal degradation [41] and to occupy specific chromatin sites of Wnt/β-catenin target genes such as cyclin D1 and c-Myc [42]. These findings indicate that hTERT depletion may alter cell cycle progression independently of telomere length in K-562 cells, through regulation of the Wnt/β-catenin or c-Myc signaling pathways.

Consistent with the downregulation of BCR-ABL protein, we reported that imatinib led to reduction of phosphorylated BCR-ABL tyrosine 177 (Y177) levels in K-562 hTERT cells. Through recruitment of growth factor receptor bound protein 2 (GRB2) and GRB2-associated binding protein 2, the auto-phosphorylated BCR-ABL Y177 activates Ras-mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)-Akt-signal pathways resulting in regulation of numerous proteins involved in cell survival and proliferation [43,44]. In particular, the PI3K/Akt cascade may modulate cyclin D1 [45,46], c-Myc [47] as well as p21 and p27 [48] expression. In our study, concomitant to the decreased phosphorylation of BCR-ABL Y177, we provided evidence that imatinib leads to alterations of the expression levels of cell cycle-related proteins. These findings indicate that the anti-proliferative effect of imatinib is at least in part due to its impact on BCR-ABL tyrosine kinase activity. Importantly, we also demonstrated that hTERT depletion sensitizes K-562 cells to the anti-proliferative effect of imatinib, enhances the reduction of phosphorylated BCR-ABL Y177 and upregulates either p21 or p27 expression, thereby suggesting that hTERT depletion may potentiate imatinib efficacy by affecting BCR-ABL.



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Here, we showed that telomerase inhibition combined to imatinib triggers a senescence-like phenotype in K-562 cells, as shown by increased SA-β-Gal activity and lysosomal compartment, that are associated with a delay in cell cycle progression but fails to trigger permanent growth arrest. The senescence program was classically defined as an irreversible cell cycle arrest in G1 or G2/M phase and is associated with accumulation of the lysosomal enzyme β-galactosidase [49]. However, the concept of senescence as a permanent cell cycle arrest has been challenged. Indeed, Sherman et al. showed that oncogenic-induced senescent breast cancer cells did not display permanent cell cycle arrest despite increased SA-β-Gal activity and p21 expression [50]. In addition, oncogenic activation or DNA damage may trigger senescence-like features in terminally differentiated cells such as adipocytes, hepatocytes and neurons, indicating that senescence is not rigidly linked

Fig. 7. hTERT depletion combined to imatinib elicits a cellular senescence-like phenotype. (A) WT K-562 cells were treated for 72 h with 25 μM PDS and 12 μM RHPS4, two reference chemical inhibitors of telomerase activity. The positive control (Dox) corresponds to K-562 cells treated with 80 nM doxorubicin for 72 h. Upper panel: cytochemical detection of SA-ß-Gal activity. Lower panel: quantification of SA-ß-Gal activity. * and ** indicate $p < 0.05$ and $p < 0.01$ *versus* control cells, respectively (one-way ANOVA, $p = 0.005$). (B to E) WT and hTERT deficient K-562 cells lines were treated with their respective GI_{50} values of imatinib (IM; see Table 1) for 72 h (B). Upper panel: cytochemical detection of SA-ß-Gal activity. Lower panel: quantification of SA-ß-Gal-activity. * indicates $p < 0.05$ *versus* untreated cells, respectively (Student's *t*-test); £ and ££ indicate $p < 0.05$ and $p < 0.01$ *versus* WT K-562 cells, respectively (one-way ANOVA, $p = 0.007$); $ and $$ indicate $p < 0.05$ and $p < 0.01$ *versus* imatinib-treated WT K-562 cells, respectively (one-way ANOVA, $p = 0.006$). (C) Analysis of lysosomal mass with pictures representing the Hoechst stained nuclei, Lysotracker-red stained lysosomes and the merged signal. (D) Lysosomal mass quantification. * and ** indicate $p < 0.05$ and $p < 0.01$ *versus* untreated cells, respectively (Student's *t*-test); £ and ££ indicate $p < 0.05$ and $p < 0.01$ *versus* WT K-562 cells, respectively (one-way ANOVA, $p = 0.06$); $ and $$ indicate $p < 0.05$ and $p < 0.01$ *versus* imatinib-treated WT K-562 cells, respectively (one-way ANOVA, $p = 0.002$). (E) Western Blot analysis of γH2AX expression. Values of fold change relative to loading control are indicated under blots. All pictures are representative of three independent experiments and all graphs represent the mean ± SD of three independent experiments. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

to a permanent growth arrest [51–53].

Cellular senescence is considered as a tumor suppressor mechanism that prevents the proliferation of damaged cells at risk of malignant transformation [54]. Importantly, factors secreted by senescent tumor cells may attract immune cells, which in turn trigger the elimination of these cells through immune-mediated clearance, thus resulting in tumor regression [10]. Nevertheless, senescent tumor cells may promote tumorigenesis and the formation of secondary tumors or cancer relapse. Thus, persistence of therapy-induced senescent cells after treatment may be detrimental. In this context, pro-senescence therapy combined with senotherapeutics or senolytics driving senescent cells to cell death has emerged as a novel anti-cancer strategy with or without traditional chemotherapies [55].

Although BCR-ABL TKIs are very effective in the treatment of CML, these inhibitors have limited efficacy against LSCs, which are responsible for therapeutic resistance and disease progression [22]. Thus, new therapeutic strategies targeting LSCs are needed to improve the long-term outcomes of CML therapy. Since telomerase is overexpressed in LSCs and is involved in maintaining their self-renewal and replication potential [56], LSCs-targeted strategies through telomerase inhibition represents an attractive treatment for CML. Increasing evidence indicates that ALDH activity is upregulated in cancer stem cell populations and therefore has been recognized as a marker for cancer stem cells [57–60]. In this study, we reported that hTERT depletion in K-562 cells decreased the percentage of LSCs, as shown by the reduction of $ALDH^+$ cells. Recent studies demonstrated that telomerase inhibition in acute myeloid leukemia cells targets LSCs [56,61,62]. Nevertheless, it has not yet been determined whether telomerase inhibition is effective in CML LSCs. Therefore, our study is the first one revealing that telomerase inhibition may lead to a decrease of CML stem cells. Interestingly, we further showed that hTERT depletion sensitizes CML stem cells to imatinib treatment.

Recently, several studies reported that ALDH1 is transcriptionally regulated by β-catenin together with T cell factor (TCF)/lymphoid enhancer factor (LEF) in prostate [63], breast [64,65] and ovarian [66] cancers. In addition, Park et al. found that hTERT modulates the β-catenin signaling pathway by acting as a cofactor in a β-catenin transcriptional complex [42]. These findings suggest that ALDH could be regulated by hTERT depletion in K-562 cells, in our study.

β-catenin/TCF activity are potentially regulated by different mechanisms including regulation of β-catenin stability and recruitment of co-repressors or co-activators. Notably, BCR-ABL stabilizes β-catenin through its phosphorylation, thereby triggering its translocation into the nucleus, where β-catenin binds to TCF/LEF family transcription factors and regulates the expression of a large number of target genes including cyclin D1 and c-Myc [67,68]. Thus, we hypothesize that potentiation of imatinib-induced $ALDH^+$ downregulation by hTERT depletion could be mediated by modulation of β-catenin/TCF transcriptional complex through BCR-ABL and hTERT.

Treatment-free remission (TFR) after discontinuation of TKI therapy has emerged as a promising management strategy for CML patients to reduce side effects associated with long-term TKI therapy, improve patients' quality of life and decrease the healthcare cost burden [69]. Clinical trials demonstrated that about half of the patients who achieved a sustained and deep molecular response remain in TFR after discontinuing TKI therapy [70,71]. Recent evidence indicated an association between telomere length shortening and TFR upon TKI discontinuation. Authors suggested that relapse upon TKI discontinuation may be due to the persistence of CML LSCs that harbor longer telomeres and escape senescence or apoptosis through telomerase upregulation after TKI therapy [72]. These data highlighted that telomere length shortening and decreased hTERT expression could be considered as predictive factors for successful TFR. In this context, combination of TKI therapy with hTERT inhibition could be an interesting option to increase TFR rate through targeting the LSC reservoir in CML patients.

5. Conclusions

Altogether, our study reveals for the first time the therapeutic potential of telomerase inhibition combined with imatinib in CML to eliminate tumor cells and prevent relapse by targeting LSCs and thus to improve the long-term outcome of CML patients.

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Authors' contributions

CG: conceptualization, methodology, validation, formal analysis, investigation, writing - original draft, writing - review & editing, visualization, project administration;

MS: conceptualization, supervision, writing - review & editing.

AG and DG: formal analysis;

CC: formal analysis;

AM: methodology, writing - review & editing;

MDic: supervision, writing - review & editing, project administration, funding acquisition;

MDie: conceptualization, supervision, writing - review & editing, project administration, funding acquisition.

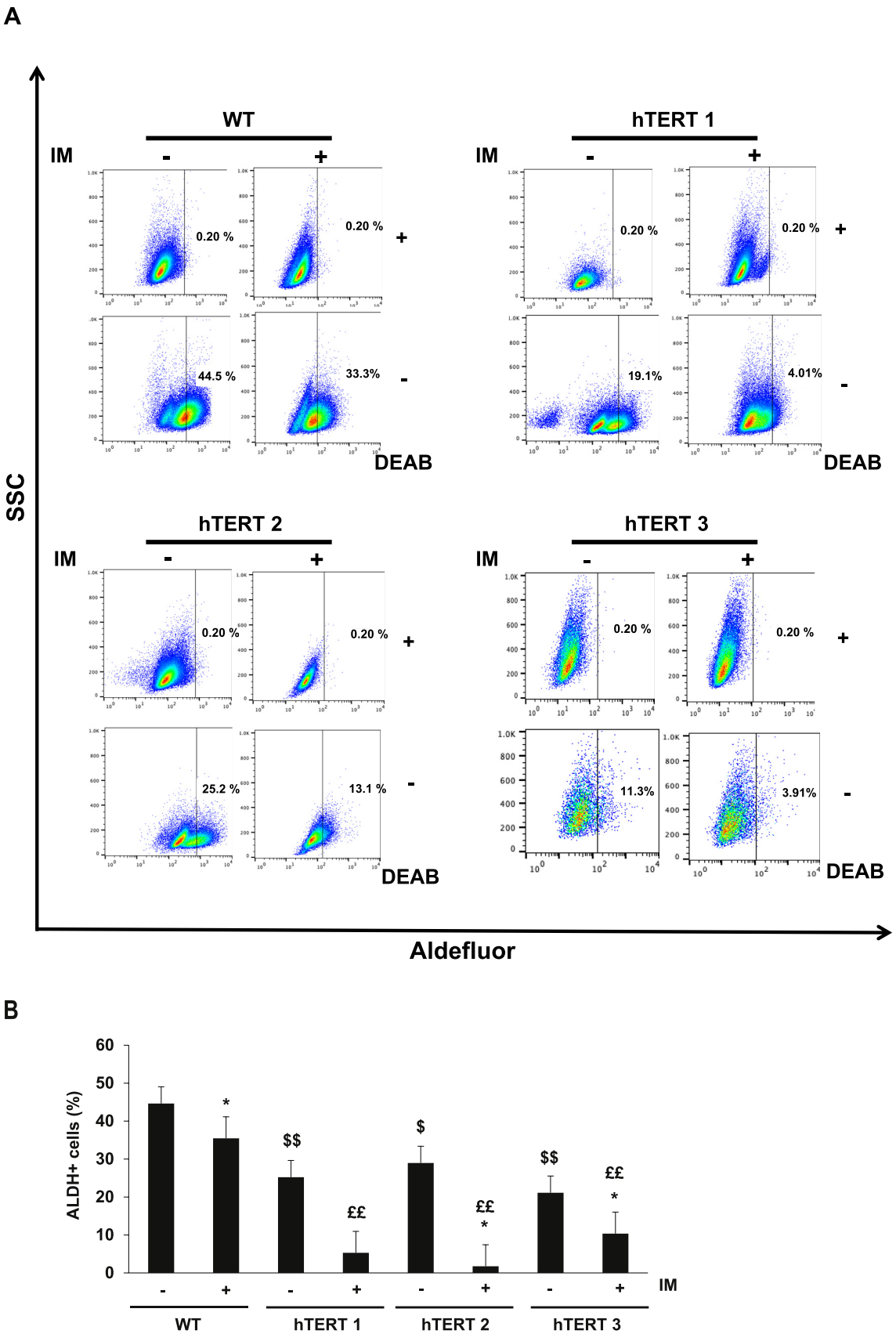

Fig. 8. hTERT depletion enhances imatinib-induced cancer stem cell population reduction in CML cells. WT and hTERT-deficient K-562 cells were exposed to their respective GI_{50} values of imatinib (IM; see Table 1) and the analysis of ALDH activity was performed after 72 h of treatment. (A) Representative aldefluor fluorescence *versus* side scatter signal (SSC) dot blots in the presence or absence of the ALDH inhibitor, diethylaminobenzaldehyde (DEAB). Baseline fluorescence was determined using DEAB-treated samples and the percentage of ALDH + cells is indicated. (B) Percentage of $ALDH^+$ cells. Results represent the mean ± SD of three independent experiments. * indicates $p < 0.05$ *versus* WT K-562 cells (Student's *t*-test); £ and ££ indicate $p < 0.05$ and $p < 0.01$ *versus* untreated cells, respectively (one-way ANOVA, $p = 0.004$); $ and $$ indicate $p < 0.05$ and $p < 0.01$ *versus* imatinib-treated WT K-562 cells, respectively (one-way ANOVA, $p < 0.001$).

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.11.017.

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